

1-2015

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# Quantitation of ortho-cresyl phosphate adducts to butyrylcholinesterase in human serum by immunomagnetic-UHPLC-MS/MS

Darryl Johnson,<sup>a</sup> Melissa D. Carter,<sup>b\*</sup> Brian S. Crow,<sup>b</sup> Samantha L. Isenberg,<sup>a</sup> Leigh Ann Graham,<sup>a</sup> H. Akin Erol,<sup>a</sup> Caroline M. Watson,<sup>a</sup> Brooke G. Pantazides,<sup>b</sup> Marcel J. van der Schans,<sup>c</sup> Jan P. Langenberg,<sup>c</sup> Daan Noort,<sup>c</sup> Thomas A. Blake,<sup>b</sup> Jerry D. Thomas<sup>b</sup> and Rudolph C. Johnson<sup>b</sup>



Tri-ortho-cresyl phosphate (ToCP) is an anti-wear, flame retardant additive used in industrial lubricants, hydraulic fluids and gasoline. The neurotoxic effects of ToCP arise from the liver-activated metabolite 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (cresyl saligenin phosphate or CBDP), which inhibits esterase enzymes including butyrylcholinesterase (BChE). Following BChE adduction, CBDP undergoes hydrolysis to form the aged adduct ortho-cresyl phosphoserine (oCP-BChE), thus providing a biomarker of CBDP exposure. Previous studies have identified ToCP in aircraft cabin and cockpit air, but assessing human exposure has been hampered by the lack of a laboratory assay to confirm exposure. This work presents the development of an immunomagnetic-UHPLC-MS/MS method for the quantitation of unadducted BChE and the long-term CBDP biomarker, oCP-BChE, in human serum. The method has a reportable range from 2.0 ng/ml to 150 ng/ml, which is consistent with the sensitivity of methods used to detect organophosphorus nerve agent protein adducts. The assay demonstrated high intraday and interday accuracy ( $\geq 85\%$ ) and precision ( $RSD \leq 15\%$ ) across the calibration range. The method was developed for future analyses of potential human exposure to CBDP. Analysis of human serum inhibited *in vitro* with CBDP demonstrated that the oCP-BChE adduct was stable for at least 72 h at 4, 22 and 37 °C. Compared to a previously reported assay, this method requires 75% less sample volume, reduces analysis time by a factor of 20 and demonstrates a threefold improvement in sensitivity. Published 2015. This article is a U.S. Government work and is in the public domain in the USA.

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**Keywords:** tri-ortho-cresyl-phosphate; butyrylcholinesterase; cresyl saligenin phosphate; Jamaica ginger paralysis; organophosphate-induced delayed neuropathy

## Introduction

Tricresyl phosphate (TCP) is an organophosphorus additive used in a variety of applications including industrial lubricants, hydraulic fluids and gasoline.<sup>[1,2]</sup> Commercial TCP consists of a mixture of ten positional cresyl isomers.<sup>[3]</sup> The mono-, di- and tri-*ortho* isomers are considered to be the most harmful, exhibiting neurotoxic properties.<sup>[4]</sup> Tri-ortho-cresyl phosphate (ToCP) has been the most extensively studied *ortho* isomer.<sup>[5]</sup> Human exposure to ToCP has been shown to cause peripheral nerve damage and degeneration of the spinal cord.<sup>[6]</sup> The most infamous case of human exposure to ToCP occurred in the United States in 1930. Consumption of Jamaica Ginger extract ('Jake') that had been adulterated with ToCP led to a condition known as 'Jamaica Ginger Paralysis' or 'Jake Leg'. Approximately 50 000 Americans became paralyzed and for most, their paralysis was permanent.<sup>[7,8]</sup> Other widespread cases of ToCP poisoning include the consumption of adulterated cooking oil in Morocco in 1959 causing the paralysis of approximately 10 000 people, and the most recent exposure occurred in 1995 from ingestion of flour tainted with ToCP.<sup>[9,10]</sup>

Most industrial uses of TCP were discontinued by 2002; however, it is still used in jet engine lubricating oils and hydraulic fluids for its anti-wear and flame retardant properties.<sup>[11]</sup> Human exposure to

ToCP can potentially occur from aircraft bleed air systems which supply fresh air throughout the cabin. In a bleed air system, hot, compressed air is pulled from bleed ports in the compressor stage of the engine. The air is then cooled and circulated throughout the cabin. Engine seals responsible for preventing engine oil from leaking out of the compressor zone can malfunction due to age, stress or wear. If this occurs, engine oil can leak out of the compression zone and into the bleed air system.<sup>[12–14]</sup> Previous studies have identified the presence of TCP and ToCP in cabin and cockpit air, as well as in the plane's air duct system.<sup>[14–19]</sup> Other reports have focused on the effects of human exposure to contaminated cabin

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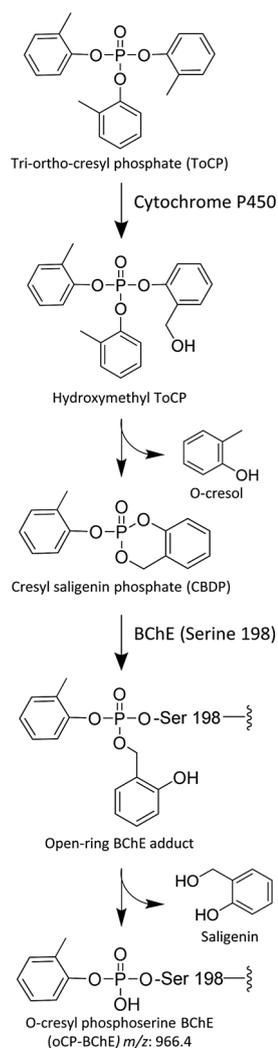
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air; however, establishing a causal link with ToCP exposure has been difficult without an analytical test with the specificity to confirm exposure.<sup>[13,20,21]</sup>

The neurotoxic effects from ToCP exposure arise from a liver-activated metabolite rather than the parent compound.<sup>[4]</sup> ToCP is metabolized to 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (cresyl saligenin phosphate or CBDP) by cytochrome P450 (Scheme 1).<sup>[22]</sup> CBDP inhibits carboxylesterases, neurotoxic esterase, acetylcholinesterase and butyrylcholinesterase (BChE).<sup>[23,24]</sup> CBDP forms a covalent adduct to the active serine site (Ser198) of BChE. A study of the kinetics of adduct formation showed that CBDP was a potent BChE inhibitor ( $k_i = 1.6 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ ).<sup>[25]</sup> As illustrated in Scheme 1, the initial adduct formed is a derivative of CBDP and within seconds, aging occurs to produce *o*-cresyl phosphoserine BChE (oCP-BChE).<sup>[25,26]</sup> The resulting +170 amu oCP-BChE adduct was identified in earlier studies following the reaction of CBDP with BChE.<sup>[24–26]</sup> This work established oCP-BChE as a potential biomarker for CBDP exposure, and by extension, ToCP exposure.

In 2011, Marsillach *et al.* demonstrated that an immunomagnetic separation could be used to purify BChE and CBDP-adducted BChE from human serum.<sup>[24]</sup> The immunomagnetic separation was later applied to a biomonitoring study of blood collected from



**Scheme 1.** Metabolism of ToCP and formation of the *o*-cresyl phosphoserine (oCP) adduct on human BChE active site serine-198.

jet airline passengers. The study monitored for the presence of phosphoserine BChE, a generic biomarker of organophosphate exposure, and low exposure levels were reported (0.05 to 3% inhibition of plasma BChE).<sup>[27]</sup> The group later developed an assay to detect the more specific adduct oCP-BChE. Improvements were also made to the BChE purification strategy to reduce sample volume, and electrospray ionization was integrated into the assay for improved reproducibility. The assay's sensitivity was reported as 0.1% oCP-BChE in blood; however, the method required lengthy analysis times making it undesirable for high-throughput analysis.<sup>[28]</sup> Until now, a high-throughput method for the quantitation of oCP-BChE adducts in clinical samples was unavailable. This work addresses this need and presents the development of a high-throughput immunomagnetic UHPLC-MS/MS method for the quantitation of oCP-BChE adducts in human blood matrices.

## Materials and methods

### Materials

The following synthetic unlabeled and stable isotopically labeled peptides were obtained from TNO (Rijswijk, The Netherlands): BChE: synthetic unlabeled butyrylcholinesterase nonapeptide (FGESAGAAS); BChE\*: isotopically-labeled BChE nonapeptide ( $^{13}\text{C}_9$ -FGESAGAAS); oCP-BChE: synthetic unlabeled *o*-cresyl phosphoserine BChE nonapeptide (FGES[oCP]AGAAS); oCP-BChE\*: isotopically labeled oCP-BChE nonapeptide ( $^{13}\text{C}_9$ -FGES[oCP]AGAAS). Peptide amino acid analysis (AAA) was performed at Midwest Bio-Tech, Inc. (Fishers, IN, USA) and determined to be the following: BChE: 68.0%; BChE\*: 62.3%; oCP-BChE: 53.3%; oCP-BChE\*: 75.7%. BChE monoclonal antibodies from clone 3E8 were purchased from ThermoFisher Affinity Bioreagents (Rockford, IL, USA). HPLC grade acetonitrile and deionized water were commercially available from Tedia (Fairfield, OH, USA). Formic acid (98%), phosphate buffered saline with Tween 20 (PBST) dry powder, dimethyl pimelimidate dihydrochloride (DMP), 0.2M tris buffered saline (TBS) 10 $\times$  concentrate, triethanolamine buffer solution and pepsin from porcine gastric mucosa were all purchased from Sigma Aldrich (St. Louis, MO, USA). Dynabeads Protein G were obtained from Life Technologies (Carlsbad, CA, USA). KingFisher 96 Flex microplates (200  $\mu\text{l}$ ), KingFisher 96 tip combs for deep well magnets, KingFisher Flex microliter deepwell 96 plates (v-bottom), protein precipitation plates, PCR foil and easy pierce 20- $\mu\text{m}$  heat sealing foil were purchased from Fisher Scientific (Rockford, IL, USA). Nonsterile MultiScreen HTS HV 0.45- $\mu\text{m}$  opaque filter plates were purchased from EMD Millipore (Billerica, MA, USA). Sample concentration used a Porvair MiniVap Blowdown Evaporator (Porvair Sciences, Wrexham Wales, UK).

### Stock solutions and quality control (QC) materials

Synthetic BChE and oCP-BChE peptide calibrators were made following amino acid content analysis. Stock solutions were made for each native and isotopically labeled standard (1.00 mg/ml) in 0.1% formic acid and stored at  $-70^\circ\text{C}$ . Native peptide stock solutions were combined and diluted in 0.1% formic acid to prepare the eight calibrators. The isotopically labeled stock solutions were combined to prepare a single internal standard solution at a concentration of 500 ng/ml. Commercial pooled human serum adducted with sarin was obtained from Battelle Memorial Institute (Columbus, OH) and served as a matrix blank. Sarin-inhibited serum was selected since it was nearly depleted of the unadducted BChE

protein (back-calculated concentration of BChE in matrix blank serum was below the method's lowest reportable limit) and did not provide interference in the detection of oCP-BChE calibrators. Quality control (QC) materials for BChE consisted of commercial unexposed pooled human serum purchased from TNO for the mid-level QC and synthetic BChE peptide spikes into matrix blank serum for low- and high-level BChE QC samples. oCP-BChE QC materials consisted of commercial pooled human serum exposed to CBDP (TNO) for the low-level QC and synthetic oCP-BChE peptide spikes into matrix blank serum for mid- and high-level QC samples. The method QC materials used blood products acquired from commercial sources, and the work did not meet the definition of human subjects as specified in 45 CFR 46.102 (f). The commercial blood products were screened for regulatory pathogens prior to commercial sale.

### Convenience sample set

A commercial convenience set of 96 individual human sera products were purchased from Tennessee Blood Services (Memphis, TN) to evaluate baseline level responses in a population in which exposure was not expected. The method used blood products acquired from commercial sources, and the work did not meet the definition of human subjects as specified in 45 CFR 46.102 (f). The commercial blood products were screened for regulatory pathogens prior to commercial sale.

### Sample preparation

Pantazides *et al.* previously described optimization of the immunomagnetic separation and pepsin digest.<sup>[29]</sup> A recovery of approximately 90% of unadducted BChE in serum was reported, and similar recoveries were observed for BChE inhibited by organophosphorus compounds similar to oCP- (GB and VX). Pepsin digestion parameters were also optimized, reporting that the BChE protein is fully digested into the nonapeptide (FGES<sub>198</sub>AGAAS) within 30 min. Synthetic native and isotopically labeled nonapeptides were stable in these digestion conditions for up to 2 h.<sup>[29]</sup>

Samples were prepared as described by Pantazides *et al.*,<sup>[29]</sup> with a modification made to the immunomagnetic purification. Incubation time was reduced to 45 min, which did not alter protein extraction efficiency. Briefly, immunomagnetic beads were prepared by applying a DynaMag-15 magnet to 2 ml of Dynabeads Protein G. The supernatant was removed, and beads were resuspended in 4 ml of PBST. After vortex mixing, the PBST was removed and discarded. This wash step was repeated twice. Magnetic beads were then resuspended in 8 ml PBST and combined with 400  $\mu$ g of BChE monoclonal antibody. The mixture was subjected to rotation on a Dynal Sample Mixer (speed 20) overnight at room temperature. The following day, the supernatant was removed, and 4 ml aliquots of triethanolamine buffer were added to the magnetic beads. The triethanolamine buffer was removed and discarded, and this wash step was repeated once. A 4 ml aliquot of 27 mg DMP in 5 ml triethanolamine buffer was then added to the beads and allowed to rotate (speed 20) on the Dynal Sample Mixer for 30 min at room temperature. The supernatant was then removed, and magnetic beads were resuspended in 4 ml of TBS. After 15 min of rotation (speed 20) at room temperature, TBS was removed and discarded. The magnetic beads were then washed twice in 2 ml of PBST. Following two PBST washes, 1.9 ml aliquots of PBST were added to the beads. The magnetic bead suspension was stored at 4 °C until use or up to 3 months.

Following magnetic bead preparation, 125  $\mu$ l aliquots of serum (either matrix blank, QC for BChE or QC for oCP-BChE) were pipetted into a multiscreen HTS HV 0.45  $\mu$ m opaque filter plate and centrifuged at 3000 *g* for 5 min at 20 °C to remove any fibrous tissue. After centrifugation, 75  $\mu$ l aliquots of filtered serum were transferred to a 96-well KingFisher deep-well plate. To wells receiving calibrators, 75  $\mu$ l aliquots of matrix blank were added to address potential matrix effects. A plate containing BChE antibody-conjugated magnetic beads was prepared by adding 50  $\mu$ l aliquots of beads into all corresponding serum-containing wells of a 96-well KingFisher shallow-well plate. A ThermoScientific KingFisher Flex magnetic particle processor was used to transfer the BChE antibody-conjugated magnetic beads to the wells containing filtered serum. BChE protein was bound to antibody-conjugated magnetic beads by shaking with an Eppendorf MixMate at 1400 rpm for 45 min at room temperature. The KingFisher was then used to transfer the protein-bound beads through three deep-well wash plates containing 500  $\mu$ l PBST, and then to a 96-well KingFisher shallow-well digestion plate. Each well of the digestion plate contained 10  $\mu$ l of 2 mg/ml pepsin and 10  $\mu$ l of 500 ng/ml isotopically labeled internal standard solution to give a final internal standard concentration of 58.8 ng/ml for BChE\* and oCP-BChE\*. The digestion plate also contained 75  $\mu$ l of calibrator solution (2, 4, 8, 16, 32, 63, 125 or 150 ng/ml of BChE and oCP-BChE synthetic peptides) or 75  $\mu$ l of 0.6% formic acid for QC and matrix blank samples. The digestion plate was maintained at 37 °C and mixed at 1000 rpm for 10 s per min for 30 min on an Eppendorf Thermomixer. Following digestion, the KingFisher was used to remove beads from the sample. Acetonitrile aliquots of 285  $\mu$ l were added to a 0.2  $\mu$ m filter, Pierce 2 ml protein precipitation plate. The digested samples were then manually added to the protein precipitation plate. A vacuum manifold was used to filter the samples through the protein precipitation plate and into a 96-well KingFisher deep-well plate. Samples were then dried to completeness under nitrogen pressure at 60 °C. Dried samples were resuspended in 75  $\mu$ l of 0.6% formic acid for MS analysis.

### UHPLC-MS/MS

BChE and oCP-BChE levels were determined in human serum using an Agilent 1290 Infinity LC system coupled with an Agilent 6490 Triple Quadrupole mass spectrometer (Agilent, Santa Clara, CA). The mass spectrometer utilized a jet stream ionization interface, with sample analysis conducted in positive ionization mode. The instrument was tuned and calibrated bimonthly over a mass range of *m/z* 50–1400, using the Agilent ESI tuning mixture (P/NG1969). Samples (3  $\mu$ l) were injected onto a Waters Acquity UPLC HSS PFP column (1.8  $\mu$ m, 1.0 mm  $\times$  50 mm) (Waters, Milford, MA). Column compartment temperature was set to 60 °C, and autosampler temperature was 10 °C. Mobile phases consisted of 0.1% formic acid in (A) water and (B) acetonitrile. Gradient conditions increased mobile phase B concentration from 2% to 60% over 1.33 min, followed by a reequilibration of the chromatograph at 2% B for 0.66 min, producing a total analysis time of 2 min per sample. Flow rate was held constant at 300  $\mu$ l/min. The following parameters were used with the jet stream ionization source: drying gas temperature = 225 °C; drying gas flow = 11 l/min; nebulizer = 60 psi; sheath gas temperature = 350 °C; sheath gas flow = 11 l/min; capillary voltage = 6000 V; nozzle voltage = 2000 V; iFunnel high pressure RF = 210 V; iFunnel low pressure RF = 120 V; fragmenter voltage = 250 V. The mass spectrometer was operated in dynamic multiple reaction monitoring mode at 'unit' resolution of 0.7 amu full width at half-maximum height,

with a cycle time of 100 ms. Detection of BChE peptides used the following parameters: BChE quantitation ion  $m/z$  796.3  $\rightarrow$  691.3, collision energy = 25 V, cell accelerator voltage = 2.5 V. BChE confirmation ion  $m/z$  796.3  $\rightarrow$  620.3, collision energy = 27 V, cell accelerator voltage = 2.5 V. BChE\*  $m/z$  805.4  $\rightarrow$  700.3, collision energy = 25 V, cell accelerator voltage = 2.5 V. Detection of oCP-BChE peptides used the following parameters: oCP-BChE quantitation ion  $m/z$  966.4  $\rightarrow$  778.3, collision energy = 34 V, cell accelerator voltage = 5 V. oCP-BChE confirmation ion  $m/z$  966.4  $\rightarrow$  673.3, collision energy = 36 V, cell accelerator voltage = 7 V. oCP-BChE\* standard  $m/z$  975.4  $\rightarrow$  787.4, collision energy = 34 V, cell accelerator voltage = 5 V.

### Data acquisition and processing

Data were acquired using MassHunter Workstation Software, LC/MS Data Acquisition for 6400 Series Triple Quadrupole v. B.06.00, Build 6.0.6025.3 SP3. Spectral analysis and quantitation were carried out utilizing MassHunter Workstation Software Quantitative Analysis v. B.06.00 SP01, build 6.0.388.1. Accuracy was reported as percent relative error, % RE, where  $C_e$  is the experimental concentration determined from the calibration curve, and  $C_t$  is the theoretical concentration:

$$\% RE = \frac{C_e - C_t}{C_t} \times 100$$

The percentage relative standard deviation, % RSD, was calculated as a measure of assay precision, where  $C_{avg}$  is the average calculated concentration and SD is the standard deviation.

$$\% RSD = \frac{SD}{C_{avg}} \times 100$$

Peak area ratios of BChE/BChE\* and oCP-BChE/oCP-BChE\* peptides were plotted against the expected concentration to construct calibration curves from eight BChE and oCP-BChE peptide calibrators in matrix blank serum. Each calibrator was injected ( $n=32$ ) and validated over the range of 2.0–150 ng/ml. QC material characterization was completed over the course of 4 and a half weeks during method validation ( $n=32$ ) and performed by five laboratory analysts.

### Safety considerations

The analysis of BChE and oCP-BChE peptides posed no greater risk to analysts than general peptide analyses. Universal safety precautions were followed for handling biological specimens such as blood products.

## Results and discussion

### Detection and separation

Fragmentation of synthetic BChE and BChE\* peptides resulted in the predominant product ions  $m/z$  691.3 and 700.3, respectively (Fig. 1A and Supplemental Fig. 1A). Likewise, fragmentation of synthetic oCP-BChE and oCP-BChE\* peptides yielded the product ions  $m/z$  778.3 and 787.4 (Fig. 1B and Supplemental Fig. 1B). Quantitation of BChE peptides was based on the transition  $m/z$  796.3  $\rightarrow$  691.3 and confirmation by transition  $m/z$  796.3  $\rightarrow$  620.3. BChE\* peptides were analyzed monitoring the transition  $m/z$  805.4  $\rightarrow$  700.3. For oCP-BChE peptides, quantitation was based on the transition  $m/z$  966.4  $\rightarrow$  778.3. This fragment was the result of  $\beta$ -elimination of oCP from Ser-198, yielding a dehydroalanine. To confirm the

presence of oCP-BChE, the transition  $m/z$  966.4  $\rightarrow$  673.3 was used, resulting from the loss of cresyl phosphate and collision induced fragmentation to the b8 ion. oCP-BChE\* peptides were measured with transition  $m/z$  975.4  $\rightarrow$  787.4. A similar fragmentation pattern was previously reported for organophosphorus nerve agent adducts to BChE.<sup>[30]</sup> In-source fragmentation was not observed for BChE, BChE\*, oCP-BChE or oCP-BChE\* peptides.

A linear gradient of increasing mobile phase B concentration from 2% to 60% B over 1.3 min was used for the reversed-phase UHPLC separation. Under these conditions the unadducted BChE peptide was retained for 0.66 min and the oCP-BChE peptide for 0.81 min. To account for matrix effects expected in clinical sample analysis, calibrators were processed in a matrix blank serum with no reportable amounts of unadducted BChE (back-calculated concentration of BChE in matrix blank serum was below the method's lowest reportable limit). Extracted ion chromatograms collected for the matrix blank, lowest calibrator and highest calibrator for each analyte are presented (Fig. 2). The peak signal intensity of the lowest calibrator was 3 times higher than the matrix blank for the BChE peptide and 5 times higher for the oCP-BChE peptide.

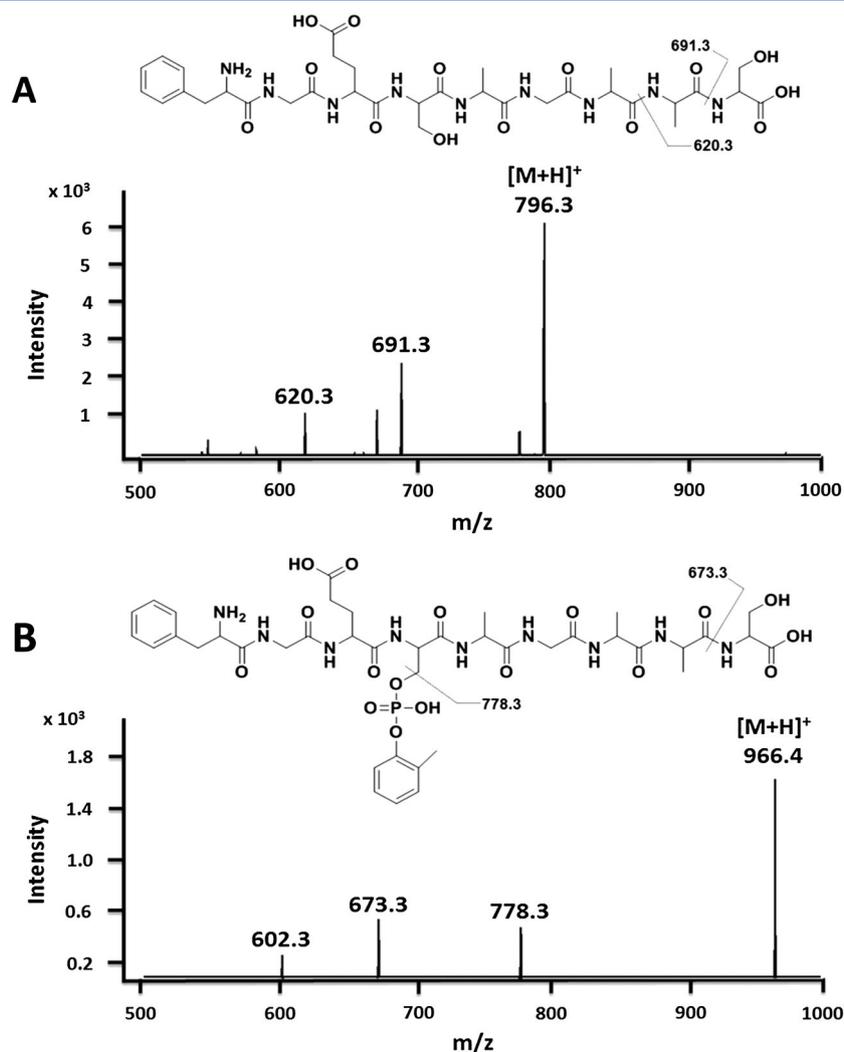
To demonstrate the applicability of this assay for clinical sample analysis, extracted ion chromatograms of uninhibited human serum and human serum exposed to CBDP are presented in Supplemental Figures. The extracted ion chromatogram of unexposed BChE is shown, and no false-positive identification of oCP-BChE is observed (supplemental Fig. 2A). An extracted ion chromatogram of human serum spiked with CBDP (supplemental Fig. 2B) shows the presence of the oCP-BChE peptide.

### Linearity

The peak area ratios of BChE/BChE\* peptides were linearly proportional to the concentration of unadducted BChE over the range of 2.0–150 ng/ml with a coefficient of determination of  $R^2 = 0.9934$  and a line equation of  $y = 0.0090x (\pm 0.0005) + 0.0064 (\pm 0.0047)$ . Likewise, the peak area ratios of oCP-BChE/oCP-BChE\* peptides were linearly proportional over the concentration range of 2.0–150 ng/ml. The reportable coefficient of determination of  $R^2 = 0.9927$  with a line equation of  $y = 0.0407x (\pm 0.0069) - 0.0237 (\pm 0.0195)$ . The reportable concentration range is consistent with methods used in the quantitation of organophosphorus nerve agent adducts to BChE in clinical samples and the abundance of unadducted BChE in the general population (40–80 nM in plasma).<sup>[29–33]</sup> The Taylor calculation was used to determine the theoretical limit of detection (LOD) for BChE at 1.19 ng/ml.<sup>[34]</sup> For oCP-BChE, the theoretical LOD was 1.89 ng/ml. The lowest calibrator for this method, 2.0 ng/ml, was the lowest reportable limit for both analytes (see Fig. 2). This corresponded to an on-column mass of 6 pg based on a 3  $\mu$ l injection volume. In 2014, Schopfer *et al.* reported a LOD of 4.0 ng/ml for oCP-BChE with a 5  $\mu$ l injection volume.<sup>[28]</sup> Based on the on-column injection mass, our method demonstrated a greater than threefold improvement in sensitivity. The enhanced sensitivity could be attributed to an efficient BChE purification technique and the use of a triple quadrupole mass spectrometer operated in multiple reaction monitoring mode.

### Recovery and matrix effects

Ideally, an isotopically labeled protein would be used to measure losses occurring during sample preparation; however, a protein



**Figure 1.** Chemical structures and product ion mass spectra of the precursor ions of synthetic peptides (A) BChE ( $[M+H]^+$   $m/z$  796.3); (B) oCP-BChE ( $[M+H]^+$   $m/z$  966.4). Fragmentation spectra were collected scanning the mass range of  $m/z$  500–1000, using a collision energy of 27 V for BChE and 34 V for oCP-BChE. The dashed lines for each structure denote the proposed sites of fragmentation. The peptide sequence shown is FGESAGAAS.

standard is not currently available. Calibrator and internal standard peptides were added at the earliest possible step since anti-BChE beads would not capture calibrators. To assess the extraction efficiency of BChE and oCP-BChE peptides during sample preparation, calibrators were added to matrix blank serum either before pepsin digestion ('Processed') or immediately prior to UHPLC-MS/MS analysis ('Unprocessed'). The 'Processed' samples were representative of the sample preparation technique used for peptide calibrators in this analysis. The average peak areas ( $n=4$ ) from processed and unprocessed samples were used to calculate recovery. As shown in Table 1, recovery for both analytes was measured at low, mid and high calibrator levels. The mean percentage recovery was  $\geq 65\%$  for BChE and  $\geq 70\%$  for oCP-BChE peptides. A few potential sources of sample loss include nonspecific pepsin digestion and peptide adsorption to plastic surfaces.<sup>[35]</sup>

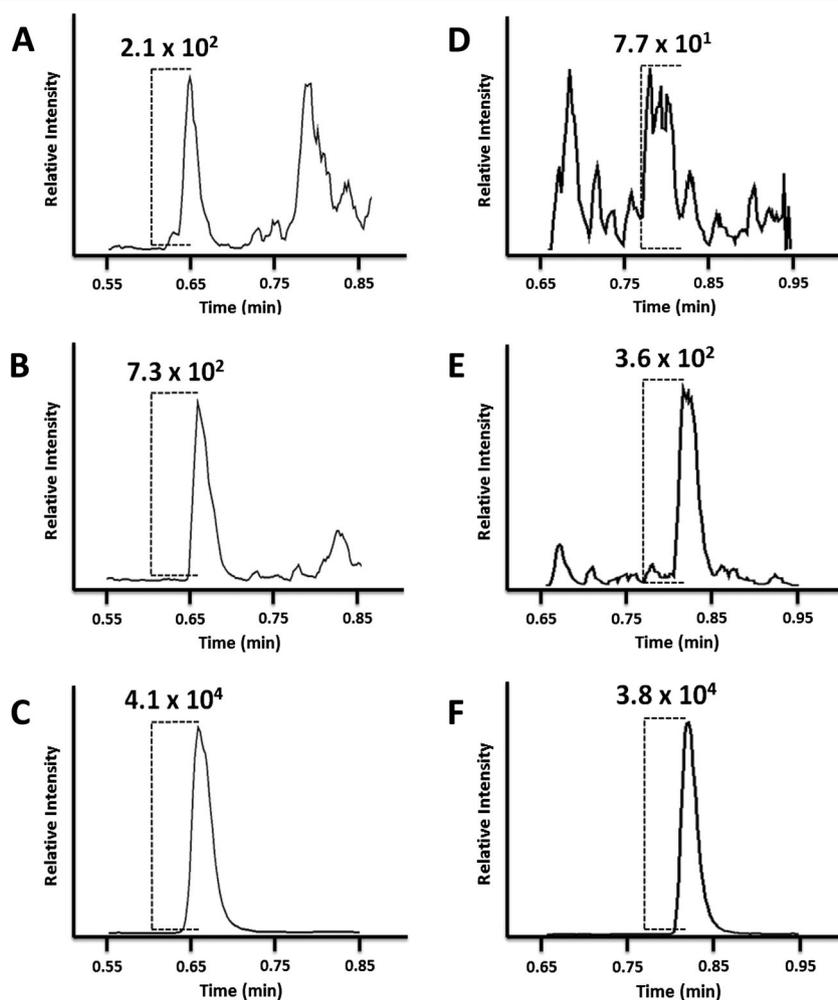
Matrix effects were evaluated by comparing the average peak areas of 'unprocessed' calibrators in matrix blank serum and in solvent solution. Matrix effects for BChE were evaluated using the highest calibrator (150 ng/ml) so that the contribution of endogenous BChE in the matrix blank would be less than 1% of the peak area. The ion suppression of BChE from matrix effects was

determined to be 8%. For oCP-BChE, matrix effects accounted for an approximate 17% reduction in mean peak area.

### Stability and ruggedness

The effects of storage were evaluated by measuring peak area ratios of BChE/BChE\* peptides and oCP-BChE/oCP-BChE\* peptides following cycles of freeze–thaws from  $-70^\circ\text{C}$  to  $22^\circ\text{C}$ . Synthetic BChE and oCP-BChE calibrators were subjected to 13 freeze–thaw cycles in order to evaluate long-term storage and use. Both BChE and oCP-BChE peptides were found to be stable  $\pm 10\%$  of theoretical concentration. Stability of internal standards was also evaluated, with BChE\* and oCP-BChE\* peptides subjected to 50 freeze–thaw cycles. The peak area of each internal standard was measured and found to be stable with a %RSD  $\leq 10\%$ .

To assess temperature effects on the analytical response ratio of BChE and oCP-BChE calibrators, peptides were allowed to stand for 0, 4, 8 and 24 h at 4, 22 and  $37^\circ\text{C}$  prior to UHPLC-MS/MS analysis. The average response ratios ( $n=4$ ) obtained at successive time points are presented in Fig. 3. All mean values were within  $\pm 10\%$



**Figure 2.** Extracted ion chromatograms of matrix blank serum containing (A) No synthetic BChE peptide added, peak produced from minor matrix contribution; (B) 2 ng/ml BChE (6 pg injection); (C) 150 ng/ml BChE (450 pg injection); (D) No synthetic oCP-BChE peptide added; (E) 2 ng/ml oCP-BChE (6 pg injection); (F) 150 ng/ml oCP-BChE (450 pg injection). Detection of BChE was based on the transition  $m/z$  796.3  $\rightarrow$  691.3. oCP-BChE used transition  $m/z$  966.4  $\rightarrow$  778.3, and quantitation was calculated from the peak area response ratio of BChE/BChE\* or oCP-BChE/oCP-BChE\*. The dashed lines indicate the chromatographic peak height.

**Table 1.** Recovery of BChE and oCP-BChE from serum at low-, mid- and high-level calibrators added prior to pepsin digestion ('processed') or immediately before UHPLC-MS/MS analysis ('unprocessed')

Calibrator (ng/ml)	Processed		Unprocessed		% Recovery <sup>a</sup>	$\sigma^b$
	Mean area	% RSD	Mean area	% RSD		
BChE						
2.0	863 ( $\pm$ 97)	11	1178 ( $\pm$ 93)	7.9	73	0.1
16	4871 ( $\pm$ 526)	11	7440 ( $\pm$ 980)	13	65	0.2
150	48 391 ( $\pm$ 2327)	4.8	70 169 ( $\pm$ 12 022)	17	69	0.2
oCP-BChE						
2.0	440 ( $\pm$ 27)	6.0	625 ( $\pm$ 69)	11	70	0.1
16	3683 ( $\pm$ 584)	16	5089 ( $\pm$ 317)	6.2	72	0.2
150	45 424 ( $\pm$ 3707)	8.2	58 291 ( $\pm$ 1757)	3.0	78	0.1

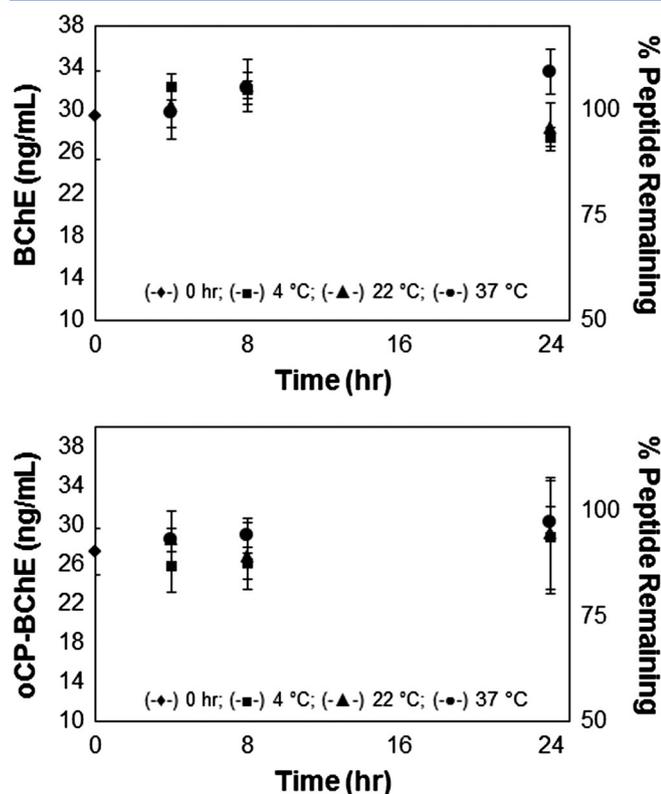
<sup>a</sup>% Recovery = [(mean processed area) / (mean unprocessed area)]  $\times$  100.

<sup>b</sup> $\sigma$  % Recovery =  $[(\sigma_{un} / \text{mean}_{un})^2 + (\sigma_{proc} / \text{mean}_{proc})^2]^{1/2}$ .

Mean and standard deviation of processed and unprocessed samples are calculated from the peak area of the processed or unprocessed ( $n = 4$  for each).

of the initial value for up to 24 h at all temperatures for BChE and oCP-BChE; however, variability of the oCP-BChE calibrator does increase at the 24-h mark (RSD approaching 20%), indicating that peptides should be stored at or below  $-20^\circ\text{C}$  when not in use.

The effects of storage were also evaluated for QC materials. Unexposed pooled serum (QC mid for BChE) and CDBP-inhibited serum (QC low for oCP-BChE) were allowed to stand for 0, 4, 8, 24, 48 and 72 h at 4, 22 and  $37^\circ\text{C}$  prior to UHPLC-MS/MS analysis. In



**Figure 3.** Stability of BChE and oCP-BChE peptide calibration standards in 0.1% formic acid buffer at 4, 22 and 37 °C. The peptides were incubated at the desired temperature for 0, 4, 8 and 24 h. Following incubation, peptides were added to matrix blank serum just prior to pepsin digestion. The calculated BChE and oCP-BChE concentrations and % peptide remaining is plotted ( $n = 4$ ); error bars represent  $\pm$ SD.

unexposed pooled serum, BChE was found to be stable at all three temperatures for at least 72 h. The mean BChE concentration at each time/temperature point ( $n = 4$  for each) was found to be within two standard deviations of the BChE concentration determined during QC characterization (see Accuracy and precision). For CBDP-inhibited serum, oCP-BChE was found to be stable at all temperatures for at least 72 h. The mean oCP-BChE concentration was within two standard deviations of the oCP-BChE concentration determined during QC characterization (see Accuracy and precision) for each time/temperature point ( $n = 4$  for each). A previous study stated the oCP-BChE adduct is unstable; however, our results demonstrate oCP-BChE in serum is stable for at least 3 days at 4, 22 and 37 °C.<sup>[36]</sup> Differences in sample preparation and analysis techniques could account for differences in adduct stability.

Ruggedness testing was conducted by evaluating changes in the following analysis parameters: column temperature, collision energy, slope of the LC gradient, LC flow rate and analytical column lot (three different product lots tested). Evaluation was based on the calculated concentration accuracy for each analyte when ruggedness parameters were individually changed to  $\pm 10\%$  of the optimized value. For example, the assay's 60 °C column temperature was also evaluated at 54 °C and 66 °C. Accuracy was found to be within  $\pm 15\%$  of the theoretical concentration when column temperature, LC gradient slope and LC flow rate were examined above and below the method's optimized settings. The use of LC columns from multiple product lots showed no impact in assay accuracy. Changes to the collision energies of each analyte did show significant impact on method accuracy with error exceeding 20%.

### Accuracy and precision

The intraday and interday accuracy and precision for the quantitation of BChE and oCP-BChE peptides were determined over four and a half weeks. Five analysts participated in method validation, analyzing two calibration curves and corresponding QCs per day (16 different days of analysis, 32 total runs). Data collected at the beginning, middle and end of method validation are shown for 2.0 ng/ml (low-), 16 ng/ml (mid-) and 150 ng/ml (high-) level calibrators. The intraday % Error for BChE peptides was  $\leq 7.8\%$  for the low-,  $\leq 4.1\%$  for the mid- and  $\leq 4.4\%$  for the high-level calibrators. Corresponding % RSDs were  $\leq 14\%$ ,  $\leq 9.8\%$  and  $\leq 6.9\%$  at the low-, mid- and high-level calibrators (Table 2). Likewise, the intraday % Error for oCP-BChE peptides was  $\leq 16\%$ ,  $\leq 10\%$  and  $\leq 6.0\%$  (Table 3). Intraday % RSD for oCP-BChE peptides was  $\leq 13\%$ ,  $\leq 5.8\%$  and  $\leq 8.9\%$ . Analysis of interday accuracy for BChE peptides demonstrated a % Error of  $\leq 0.9\%$ ,  $\leq 0.5\%$  and  $\leq 3.3\%$ , with corresponding interday % RSD of 9.5%, 6.8% and 3.3%. For oCP-BChE peptides, the interday % Error was  $\leq 8.9\%$ ,  $\leq 5.8\%$  and  $\leq 3.8\%$ , and the resultant interday % RSD was 9.9%, 5.4% and 4.9%. The method's accuracy and precision follow the guidelines in the FDA's guidance for bioanalytical method validation and thus show applicability for the analysis of clinical samples.<sup>[37]</sup>

A low-, mid- and high-level QC was used for each analyte covering the method's calibration range. The BChE mid-level QC was pooled uninhibited serum. Our assay determined the mean concentration of BChE in the mid-level QC was 36.2 ( $\pm 4.80$ ) ng/ml with a RSD = 13% ( $n = 32$ ). These results were in agreement with the measured BChE concentration from our analysis of a commercial convenience set and results obtained by Pantazides *et al.*<sup>[29]</sup> Synthetic BChE peptide spikes in matrix blank serum were used for QC samples near the upper and lower range of the calibration curve (accuracy  $\geq 96\%$ , RSD  $< 15\%$ ). For oCP-BChE, the low-level QC was made by exposing pooled human serum to CBDP. The mean concentration of oCP-BChE in the exposed serum sample was 12.2 ( $\pm 1.83$ ) ng/ml with a RSD = 15% ( $n = 32$ ). Synthetic peptide spikes into matrix blank serum were used as mid- and high-level oCP-BChE QCs (accuracy  $\geq 92\%$ , RSD  $< 15\%$ ). The precision of QC materials compared favorably with other reported assays monitoring organophosphorus compound adduction to BChE.<sup>[29,33,38]</sup>

### Application to commercial convenience set

A convenience set of commercially available individual serum samples was purchased to assess levels of unadducted BChE and oCP-BChE, if any. The mean BChE concentration of the 96 samples analyzed was 37.7 ng/ml ( $\pm 10.7$ ) with a range of 18.8 to 67.0 ng/ml. These results were similar to the mean BChE concentration reported by Pantazides *et al.* following analysis of 192 commercially purchased serum samples.<sup>[29]</sup> For oCP-BChE, all samples were found to be lower than the assay's lowest reportable limit with no evidence of peaks detected.

Thus far, the +170 amu BChE adduct observed in this work has only been reported following *in vitro* reaction of BChE with CBDP.<sup>[24–28]</sup> This suggests that oCP-BChE can be used as a biomarker of CBDP exposure. Previous studies have used phosphoserine BChE as a biomarker of CBDP; however, this adduct is not specific to CBDP exposure alone. For example, the organophosphorus nerve agent tabun also adducts to BChE, and acid hydrolysis can yield the same phosphoserine adduct.<sup>[39]</sup> Comparison of this method to previous reports for oCP adduct detection shows that the current work yields a greater than threefold improvement in sensitivity based on the on-column injection mass of the adducted

**Table 2.** Intraday and interday accuracy and precision for BChE calibrators in human serum

BChE Day	Intraday <sup>a</sup>			Interday <sup>b</sup>		
	AVG (ng/ml)	% Error <sup>c</sup>	% RSD	AVG(ng/ml)	% Error <sup>c</sup>	% RSD
2.0 ng/ml						
1	1.87	-6.75	4.93	2.02	0.833	9.50
15	2.03	1.5	13.2			
32	2.16	7.75	6.23	2.17 <sup>d</sup>	8.61 <sup>d</sup>	15.1 <sup>d</sup>
16 ng/ml						
1	16.7	4.06	9.77	16.1	0.417	6.76
15	15.8	-1.25	4.94			
32	15.8	-1.25	8.06	15.4 <sup>d</sup>	-3.53 <sup>d</sup>	9.58 <sup>d</sup>
150 ng/ml						
1	144	-4.33	1.48	145	-3.22	3.33
15	145	-3.33	6.83			
32	147	-2.00	0.962	153 <sup>d</sup>	1.67 <sup>d</sup>	7.09 <sup>d</sup>

<sup>a</sup>Intraday AVG, % Error and % RSD,  $n = 2$  for each day.  
<sup>b</sup>Interday AVG, % Error and % RSD,  $n = 6$  for each calibrator level (average from day 1, 15 and 32).  
<sup>c</sup> $[(AVG \text{ calculated conc.} - \text{theoretical conc.}) / \text{theoretical conc.}] \times 100$ .  
<sup>d</sup>AVG, % Error and % RSD are calculated from the complete method validation ( $n = 32$ ).

**Table 3.** Intraday and interday accuracy and precision for oCP-BChE calibrators in human serum

oCP-BChE Day	Intraday <sup>a</sup>			Interday <sup>b</sup>		
	AVG (ng/ml)	% Error <sup>c</sup>	% RSD	AVG (ng/ml)	% Error <sup>c</sup>	% RSD
2.0 ng/ml						
1	2.32	15.8	3.36	2.18	8.83	9.92
15	2.00	0.00	12.4			
32	2.22	11.0	10.8	2.42 <sup>d</sup>	21.1 <sup>d</sup>	11.5 <sup>d</sup>
16 ng/ml						
1	14.4	-10.0	1.96	15.1	-5.73	5.44
15	15.9	-0.938	5.80			
32	15.0	-6.25	3.77	14.7 <sup>d</sup>	-8.34 <sup>d</sup>	7.67 <sup>d</sup>
150 ng/ml						
1	150	0.333	0.470	156	3.78	4.87
15	158	5.00	1.35			
32	159	6.00	8.89	154 <sup>d</sup>	2.37 <sup>d</sup>	5.63 <sup>d</sup>

<sup>a</sup>Intraday AVG, % Error and % RSD,  $n = 2$  for each day.  
<sup>b</sup>Interday AVG, % Error and % RSD,  $n = 6$  for each calibrator level (average from day 1, 15 and 32).  
<sup>c</sup> $[(AVG \text{ calculated conc.} - \text{theoretical conc.}) / \text{theoretical conc.}] \times 100$ .  
<sup>d</sup>AVG, % Error and % RSD are calculated from the complete method validation ( $n = 32$ ).

peptide. This method also required 75% less sample volume than the previously reported methods.<sup>[27,28]</sup> This is significant when sample volume is limited, such as in the analysis of pediatric samples.

## Conclusions

This method was developed to address the current need for a quantitative method to confirm human exposure to CBDP in clinical

samples using the biomarker oCP-BChE. The oCP adduct was not observed in the commercial convenience set analyzed and to-date has only been identified following the *in vitro* reaction of BChE with CBDP. The immunomagnetic-UHPLC-MS/MS method provides quantitation of both unadducted BChE and oCP-BChE over a concentration range of 2.0 to 150 ng/ml. The sensitivity of this method is consistent with similar methodologies used in the detection of organophosphorus nerve agent adducts in serum. The assay demonstrated high intraday and interday accuracy and precision of

calibration standards. Analysis of pooled uninhibited serum and CBDP-inhibited serum QC materials over four and half weeks demonstrated high assay precision in accordance with FDA guidance for bioanalytical methods. Our results also demonstrated the oCP-BChE adduct is stable in serum for at least 72 h at 4, 22 and 37°C. This work is the first quantitative high-throughput assay reported for the measurement of oCP-BChE in human serum.

## Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service or the US Department of Health and Human Services.

## Conflict of interest

The authors declare no competing financial interest.

## Acknowledgements

This work was supported by the Centers for Disease Control and Prevention, the Defense Threat Reduction Agency and the Oak Ridge Institute for Science and Education. The authors would like to thank Ms. Chariety Sapp of the CDC's Incident Response Laboratory (IRL) for dispensing convenience set serum samples prior to analysis. The authors would also like to express their gratitude for input from colleagues Professor Clement E. Furlong of The University of Washington and Professor Oksana Lockridge of The University of Nebraska Medical Center.

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## Supporting information

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